Pressure-Induced Upregulation of Preproendothelin-1 and Endothelin B Receptor Expression in Rabbit Jugular Vein In Situ

Implications for Vein Graft Failure?

Manfred Lauth, Marc-Moritz Berger, Marco Cattaruzza, Markus Hecker

Abstract—Upregulation of endothelin-1 (ET-1) synthesis in venous bypass grafts in response to arterial levels of blood pressure may play a major role in graft failure. To investigate this hypothesis, isolated segments of the rabbit jugular vein were perfused at physiological (0 to 5 mm Hg) and nonphysiological (20 mm Hg) levels of intraluminal pressure. As judged by reverse transcription–polymerase chain reaction analysis (mRNA level), neither endothelin-converting enzyme nor endothelin A receptor expression appeared to be pressure sensitive. In contrast, there was a profound and time-dependent increase in endothelial prepro-ET-1 mRNA and intravascular ET-1 abundance (by ELISA) as well as in smooth muscle endothelin B receptor mRNA and functional protein (by superfusion bioassay) on raising the perfusion pressure from 5 to 20 mm Hg, but not from 0 to 5 mm Hg, for up to 12 hours. Video microscopy analysis revealed that the segments were distended by 75% at 5 mm Hg and near maximally at 20 mm Hg compared with the resting diameter at 0 to 1 mm Hg. Treatment of the segments with actinomycin D (1 μmol/L), the specific protein kinase C inhibitor, Ro 31–8220 (0.1 μmol/L), or the c-Src family–specific tyrosine kinase inhibitor, herbimycin A (0.1 μmol/L), demonstrated that the pressure-induced expression of these gene products occurs at the level of transcription and requires activation of protein kinase C, but not c-Src. In venous bypass grafts such deformation-induced changes in gene expression may contribute not only to acute graft failure through ET-1–induced vasospasm but also to endothelin A receptor– and/or endothelin B receptor–mediated smooth muscle cell hyperplasia and graft occlusion. **(*Arterioscler Thromb Vasc Biol*. 2000;20:96-103.)**

Key Words: blood pressure ■ endothelin-1 ■ endothelin B receptor ■ gene expression ■ graft failure

Excessive mechanical strain after exposure to arterial levels of blood pressure could trigger the overt adaptive proliferative response of venous bypass grafts, ultimately leading to graft failure.[1] Among the various mediators implicated in this putatively stretch-induced smooth muscle cell (SMC) hyperplasia, endothelin-1 (ET-1) may play a pivotal role.[2–4]

Predominantly formed by endothelial cells, this 21–amino acid peptide is not only a powerful vasoconstrictor but also a potent mitogen for vascular SMCs. It is derived from a 212–amino acid precursor, prepro-ET-1, that is sequentially processed to big ET-1 and ET-1 by a furin-like protease and an endothelin-converting enzyme (ECE-1).[3,4] ET-1 exerts its biological effects mainly through activation of two types of G-protein–coupled receptors, the endothelin A receptor (ET_A-R) and endothelin B receptor (ET_B-R).[5] Although SMCs express both types of receptors, activation of the ET_A-R appears to predominantly modulate SMC tone and proliferation in arteries, whereas in veins, ET-1 seems to exert these effects primarily through activation of the ET_B-R.[3,5] Endothelial cells also express an ET_B-R, the activation of which promotes the release of nitric oxide and prostacyclin, thereby potentially limiting an excessive ET_A-R– and/or ET_B-R–mediated SMC stimulation by ET-1.[3,5] Thus far, it is not clear whether the receptors in endothelial cells and SMCs represent the same or subtypes of ET_B-R.[6]

Cyclic stretch has been reported to enhance ET-1 peptide synthesis and prepro-ET-1 mRNA expression in cultured endothelial cells.[7–9] In the vessel wall in situ, endothelial cells are not normally exposed to this hemodynamic force, because the bulk of the physiological increase in transmural pressure is transformed into a circumferential tensile strain that almost exclusively affects the SMCs.[10] However, in situations in which the pressure-induced distension of the vessel wall is more pronounced and/or chronically elevated, as in aortocoronary venous bypass grafts, endothelial cells may also be deformed to a significant extent.

To investigate whether a pressure-induced increase in tensile strain also affects prepro-ET-1 gene expression in the

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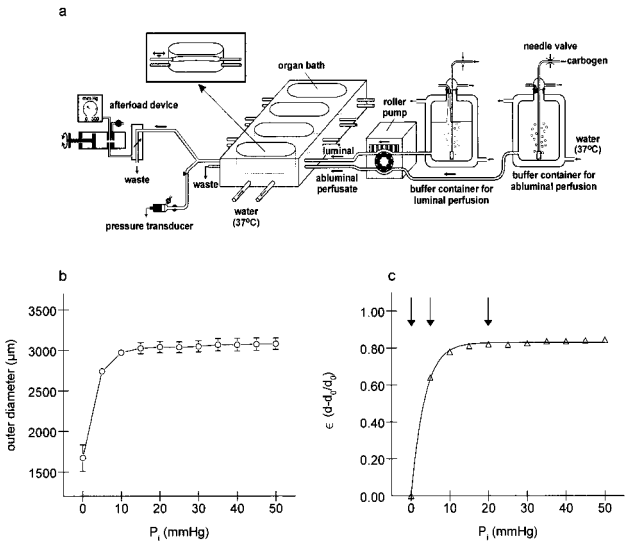

Figure 1. a, Schematic representation of the experimental setup (carbogen, 95% O_2/5% CO_2). b, Pressure-diameter relation in isolated perfused segments of the rabbit jugular vein. The figure summarizes 3 experiments performed with individual segments in which the effect of a stepwise increase in perfusion pressure (P_i) on the outer diameter of the segments was monitored by video microscopy. c, Circumferential strain (ϵ) values calculated on the basis of the mean changes in diameter shown in panel b. ϵ indicates circumferential strain; $d-d_0$, pressure induced change in diameter; and d_0, diameter at 0 mm Hg, ie, atmospheric pressure. Arrows denote the three different experimental conditions, ie, perfusion at 0 to 1, 5, and 20 mm Hg.

vessel wall in situ, we have developed an experimental model in which isolated segments of the rabbit external jugular vein are perfused at different levels of intraluminal pressure. In addition, we have investigated whether the resulting distension of the vessel wall also affects the expression of ECE-1 or that of the 2 endothelin receptors and what kind of signaling mechanism is involved therein.

Methods

Materials

3-[1-3-(Amidinothiopropyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide methane sulfonate (Ro 31−8220) was supplied by Calbiochem-Novabiochem; actinomycin D and cycloheximide, by ICN; ET-1, herbimycin A, the sarafotoxins, and the endothelin receptor antagonists, by Alexis. Acetylcholine was obtained from Sigma. 9,11-Dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) was kindly provided by The Upjohn Co.

Experimental Model

Male New Zealand White rabbits (2.0±0.1 kg body weight, n=47) were anesthetized intravenously with 60 mg/kg pentobarbital sodium (Nembutal, Sanofi) and exsanguinated by cutting through both the aorta and vena cava. The left and right external jugular veins were dissected, cut to equal size, and cleansed of adventitial adipose and connective tissue. These were cut in half, so that a set of four segments from each animal could be mounted into a specially designed 4-position perfusion chamber and stretched back to their in situ length (17.7±0.7 mm, n=74) by the aid of moveable cannulas, onto which the segments were tied (see Figure 1a). Vessel diameter was continuously monitored by video microscopy (Visitron Instruments). The lumen of the segments and the surrounding tissue baths were individually perfused (lumen 1 mL/min, bath 0.5 mL/min) with warmed (37°C) oxygenated (lumen 75% N_2/20% O_2/5% CO_2, P_{O_2} 140 mm Hg, P_{CO_2} 15 to 20 mm Hg, pH 7.4; bath 95% O_2/5% CO_2, P_{O_2} >300 mm Hg, P_{CO_2} 18 to 38 mm Hg, pH 7.4) Tyrode's solution of the following composition (in mmol/L): Na^+ 144.3, K^+ 4.0, Cl^- 138.6, Ca^{2+} 1.7, Mg^{2+} 1.0, HPO_4^{2-} 0.4, HCO_3^- 19.9, and D-glucose 10.0. An IPC roller pump (Ismatec) was used for perfusion, pumping at a frequency of 1.33 Hz with a peak pulsatile pressure of ±1 mm Hg. After an equilibration period of 30 minutes, the segments were perfused at 0, 5, or 20 mm Hg for 3 to 12 hours with the aid of an adjustable afterload device system (Hugo Sachs Elektronik). Perfusion pressure without the afterload device was monitored to be 0 to 1 mm Hg by using a pressure transducer connected to a side arm of the outflow tubing (see Figure 1a.) In experiments with drug or vehicle treatment, the segments were perfused with defined drug concentrations at a reduced flow rate (0.5 mL/min) for up to 1 hour directly after the resting phase. In another series of experiments, the segments were mechanically denuded by gentle abrasion with a roughened stainless steel cannula (2.0-mm OD) before being mounted into the perfusion chamber. To make sure that the endothelium had been successfully removed, histological, reverse transcription (RT)–polymerase chain reaction (PCR), and superfusion bioassay analyses were used (see below). At the end of the perfusion, the segments were snap-frozen in liquid N_2 and stored at −80°C.

Superfusion Bioassay

Four ring segments (3 to 4 mm wide) were tested simultaneously by mounting them between force transducers and a rigid support for measurement of isometric force (TSE). The rings were superfused at 1 mL/min with warmed (37°C), oxygenated (95% O_2/5% CO_2, P_{O_2} 400 mm Hg, P_{CO_2} 38 mm Hg, pH 7.4) Krebs-Henseleit solution of the following composition (in mmol/L): Na^+ 144.0, K^+ 5.9, Cl^- 126.9, Ca^{2+} 1.6, Mg^{2+} 1.2, $H_3PO_4^-$ 1.2, SO_4^{2-} 1.2, HCO_3^- 25.0, and D-glucose 11.1. Passive tension was adjusted over a 30-minute equilibration period to 0.4±0.1 g (n=16). Thereafter, the rings were preconstricted with 10 to 100 nmol/L U46619 to 0.9±0.1 g (n=16). To test whether the endothelium was functionally intact, 1 and 10 nmol acetylcholine was applied as a single injection (10 μL) into the superfusate during the plateau phase of constriction, and the presence or absence of a relaxant response was monitored with the aid of a digital PC–operated analysis system (Biosys, TSE). Experiments with ET-1 or the sarafotoxins were performed in the same manner, except that the segments were not actively constricted with U46619.

RT-PCR Analysis

The frozen segments were minced under liquid N_2 with the aid of a mortar and a pestle. Total RNA was isolated with the Qiagen RNeasy kit (Qiagen) followed by cDNA synthesis with a maximum of 3 μg total RNA (determined photometrically by measuring the optical density at 260 and 280 nm) and 200 U Superscript II reverse transcriptase (GIBCO, Life Technologies) in a total volume of 20 μL according to the manufacturers' instructions. For normalization of cDNA load, 5 μL of the resulting cDNA solution (corresponding to ≈75 ng) and 20 pmol of each primer (corresponding to a final concentration of 0.4 μmol/L, GIBCO) were used for elongation factor 1 (EF-1) PCR with 1 U Taq DNA polymerase (GIBCO) in a total volume of 50 μL according to the manufacturer's instructions. PCR products were electrophoretically separated on 1.5% agarose gels containing 0.1% ethidium bromide, and the intensity of the detected bands was determined densitometrically to adjust cDNA volumes for subsequent PCR analyses by using a CCD-camera system and the One-Dscan Gel analysis software (Scanalytics). PCR conditions described for EF-1 were identical for the other gene products except for the individual adjustment of cDNA volumes. All PCR reactions were performed individually for each primer pair in a Hybaid OmnE thermocycler (AWG) that was programmed as follows: a unique 2-minute period for complete denaturation at 94°C in the beginning followed by a primer-specific number of cycles of 30-second denaturation at 94°C, 30-second annealing at 53°C to 60°C (see below), and 1-minute primer extension at 72°C, with an additional 5 minutes at 72°C for final extension in the end. Individual PCR conditions were as follows (where two different sets of primers are used for detection of the same gene product [prepro-ET-1, ET_A-R, and ET_B-R], comparable results were obtained):

Prepro-ET-1: product size 517 bp, 33 cycles, annealing temperature 53°C, forward 5′ TGCTCCTGCTCCTCGCTGAT 3′, reverse 5′ AAGAGCGAGTGAGAGAGTGA 3′ (corresponding to nucleotide

sequences 270 to 289 and 786 to 767 of the rabbit prepro-ET-1 gene, GenBank accession No. X59931); product size 499 bp, 30 cycles, 58°C, forward 5′ GGAGTGTGTCTACTTCTGCCAC 3′, reverse 5′ GGGAAGAGAAAGAGCGAGTG 3′ (nucleotide sequences 296 to 317 and 795 to 776, rabbit prepro-ET-1).

ECE-1: product size 309 bp, 29 cycles, 58°C, forward 5′ GCACCCTCAAGTGGATGGAC 3′, reverse 5′ CCGGAAACACGATCTCGTTC 3′ (nucleotide sequences 1425 to 1444 and 1734 to 1715, human ECE-1, Z35307).

ET_A-R: product size 334 bp, 30 cycles, 58°C, forward 5′ CAGGGCATCCTTTTGGCTGGCACTG 3′, reverse 5′ GCGCGTTGGGGCCATTCCTCATAC 3′ (nucleotide sequences 24 to 48 and 358 to 335, human ET_AR, E07649); product size 188 bp, 30 cycles, 55°C, forward 5′ CCTTATCTACGTGGTCATTGATCT 3′, reverse 5′ AAGCCACTGCTCTGTACCTG 3′ (nucleotide sequences 421 to 444 and 608 to 589, rat ET_A-R, M60786).

ET_B-R: product size 446 bp, 33 cycles, 53°C, forward 5′ GTGCTGGGGATCATCGGGAAC 3′, reverse 5′ TGAACGGGATGAAGCAAGCAG 3′ (nucleotide sequences 570 to 590 and 1015 to 995, human ET_B-R, E07650); product size 304 bp, 33 cycles, 53°C, forward 5′ TGTTGGCTTCCCCTTCATCT 3′, reverse 5′ TGGAGCGGAAGTTGTCGTAT 3′ (nucleotide sequences 1203 to 1219 and 1506 to 1487, rat ET_B-R, X57764).

EF-1: product size 951 bp, 22 cycles, 58°C, forward 5′ TGCCGTCCTGATTGTTGCTGC 3′, reverse 5′ ATCACGGACAGC GAAACGACC 3′ (nucleotide sequences 346 to 366 and 1297 to 1276, rabbit EF-1, X62245).

CD31 (platelet and endothelial cell adhesion molecule-1[PECAM-1]): product size 362 bp, 30 cycles, 56°C, forward 5′ AACTTCACCATCCAGAAGG 3′, reverse 5′ CACTGGTATTCCACGTCTT 3′ (nucleotide sequences 1207 to 1225 and 1568 to 1550, human CD31, M28526).

Inducible nitric oxide synthase (iNOS): product size 576 bp, 30 cycles, 60°C, forward 5′ CAGCTACTGGGTCAAAGACAAGAGG 3′, reverse 5′ TGCTGAGAGTCATGGAGCCG 3′ (nucleotide sequences 543 to 567 and 1118 to 1099, rabbit iNOS, U85094).

To verify the identity of the amplification products with the designed primer pairs, we cloned and sequenced the ECE-1, ET_A-R, ET_B-R, and CD31 PCR products and found a 87% to 92% homology with the published sequences of the corresponding human and rat genes. Moreover, to ensure that the PCR amplification was indeed semiquantitative (ie, in the linear phase of the exponential amplification curve), several PCR runs were performed on each set of samples from one animal to establish the adequate numbers of cycles that usually corresponded to the number indicated above.

Measurements of ET-1 Tissue Concentrations

ET-1 was extracted from the weighted segments according to the methods of Hisaki et al[11] and Moreau et al[12] with minor modifications. Briefly, the segments were individually pulverized under liquid nitrogen and incubated with 1 mL chloroform/methanol (2:1 [vol/vol]) for 18 hours at 0°C to 4°C. After addition of 0.4 mL double-distilled water, vigorous mixing, and brief centrifugation, the protein-containing interphase was applied to a preactivated 500-mg Sep-Pak Vac C18 cartridge (Waters). After washing with 1 mL of 4% glacial acetic acid (vol/vol), 5 mL double-distilled water, 1.5 mL ethyl acetate, and 24% ethanol in 4% glacial acetic acid (vol/vol), ET-1 was eluted from the cartridge with 1.5 mL of 86% ethanol in 4% glacial acetic acid (vol/vol). After removal of the solvent in an Univapo 150H Speed-Vac (Uniequip), the nearly dried residues were dissolved in 250 μL assay buffer. The concentration of ET-1 in these tissue extracts was determined by using a commercially available ELISA kit (Amersham) according to the manufacturer's instructions. Overall recovery of ET-1 was 69.4%, and interassay and intraassay variability was 15.3% and 11.7%, respectively, as determined with tissue samples to which 1 nmol/L authentic ET-1 had been added.

Statistical Analysis

Unless indicated otherwise, all data in the figures and text are expressed as mean±SEM of n observations. Statistical evaluation was performed by Student *t* test for unpaired data with the Instat for Windows statistics software package (GraphPad Software Inc). A value of $P<0.05$ was considered statistically significant.

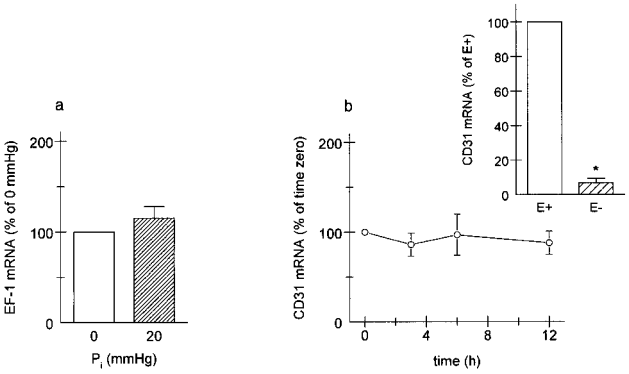

Figure 2. a, Effects of 6 hours exposure to 20 mm Hg on EF-1 mRNA abundance in non-normalized samples expressed as percentage of the mRNA level in segments from the same animal perfused at 0 to 1 mm Hg (n=20). b, CD31 mRNA abundance (expressed as percentage of the level in nonperfused segments, ie, time=0) in segments perfused at 0 to 1 mm Hg for 3 to 12 hours (n=3 to 8) as an index for endothelial cell integrity. The insert depicts the relative abundance of CD31 mRNA in endothelium-intact (E+) and endothelium-denuded (E−) segments as an index for the efficiency of endothelial cell removal (n=6). *$P<0.05$ vs E+.

Results

Control Experiments

Pressurizing of the segments to 5 mm Hg already resulted in a 75% distension of the vessel wall compared with the resting conditions (ie, 0 mm Hg), whereas the segments were almost fully distended at 20 mm Hg (Figure 1b and 1c), with an average increase in diameter from 1.7±0.1 to 4.3±0.1 mm (n=72).

Because of the rather small amounts of total RNA extractable from these segments (<3 μg), RT-PCR analysis had to be used for monitoring pressure-related changes in mRNA abundance. The mRNA level of the housekeeping reference gene, EF-1, was not altered by an increase in perfusion pressure under any of the experimental conditions described below (see Figure 2a). Moreover, there was no apparent loss of endothelial cells from the endothelium-intact perfused segments, even after 12 hours exposure to 20 mm Hg, as judged by the virtually constant mRNA level of the specific endothelial cell marker, CD31 (Figure 2b), and the maintained relaxant response to acetylcholine in the superfusion bioassay (not shown). In addition, endothelial cell integrity was checked histologically in paraffin-embedded hematoxylin/eosin-stained tissue sections of formaldehyde-fixed segments (not shown). Even though the experiments were not performed under sterile conditions, there was no microscopically visible contamination at any time point, and as judged by RT-PCR analysis, no expression of iNOS mRNA, an extremely sensitive marker for the presence of bacterial lipopolysaccharides, could be detected. On the other hand, iNOS mRNA levels were significantly upregulated after 12 to 30 hours exposure of the segments to tumor necrosis factor-α (1000 U/mL) and interferon-γ (200 U/mL, not shown).

Prepro-ET-1 Expression and ET-1 Synthesis

There was a distinct amount of prepro-ET-1 mRNA detectable in endothelium-intact and -denuded segments (95±17% of the mRNA level in intact segments, n=5) that remained

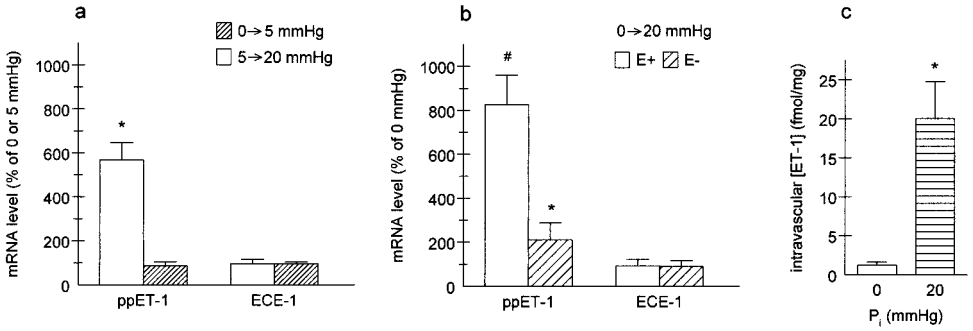

Figure 3. a, Changes in prepro-ET-1 (ppET-1) and ECE-1 mRNA abundance (expressed as percentage of the mRNA level in segments perfused at 0 to 1 or 5 mm Hg) in E+ segments after raising the perfusion pressure from 0 to 1 to 5 mm Hg for 6 hours or, after a 3-hour equilibration period at 5 mm Hg, from 5 to 20 mm Hg for 6 hours (double determination, n=3). *$P<0.05$ vs 0→5 mm Hg. b, Effects of 6 hours exposure to 20 mm Hg on the level of ppET-1 and ECE-1 mRNA in E+ and E− segments (n=5). *$P<0.05$ vs E+; #$P<0.05$ vs 0 to 1 mm Hg. c, Intravascular concentration of ET-1 (expressed as femtomoles per milligram wet weight) in E+ segments of the rabbit jugular vein perfused for 6 hours at 0 to 1 and 20 mm Hg (n=8). *$P<0.05$ vs 0 to 1 mm Hg.

virtually constant when the endothelium-intact segments were perfused at either 0 or 5 mm Hg for up to 12 hours (see Figure 3a). Prepro-ET-1 expression was significantly upregulated, on the other hand, when the segments were perfused at 20 mm Hg for 3 to 12 hours (see Figure 4a) or when they were first equilibrated at 5 mm Hg for 3 hours and then exposed to 20 mm Hg for 6 hours (Figure 3a). For reasons of simplicity, therefore, most of the experiments described below were performed by comparing segments perfused at 0 mm Hg with those perfused at 20 mm Hg.

The pressure-induced increase in prepro-ET-1 mRNA abundance seemed to occur predominantly in the endothelium, because it was strongly diminished after denudation of the segments (Figure 3b). The modest, albeit nonsignificant, increase in prepro-ET-1 mRNA abundance after 6 hours exposure to 20 mm Hg in the denuded segments, on the other hand, may have been the result of an incomplete removal of the endothelium, in view of the fact that a small amount of CD31 mRNA was still detectable in the denuded segments (Figure 2b insert).

In addition to the increase in prepro-ET-1 mRNA, the intravascular concentration of ET-1 in the endothelium-intact segments was also markedly elevated after 6 hours exposure to a perfusion pressure of 20 mm Hg (Figure 3c).

ECE-1, ET_A, and ET_B Receptor Expression

In contrast to prepro-ET-1 mRNA and ET-1 peptide, no pressure-induced increase in ECE-1 mRNA was detected in endothelium-intact or -denuded segments (Figure 3a and 3b). Raising the perfusion pressure to 20 mm Hg for 3 to 12 hours also did not significantly affect ET_A-R mRNA abundance (see Figure 4b and 4c). In contrast, ET_B-R mRNA levels were markedly increased after 3 to 12 hours exposure to 20 mm Hg (Figure 4a) but not 5 mm Hg (Figure 4b) This pressure-induced increase in ET_B-R mRNA abundance was equally detected with rather different primer pairs designed from the sequence of the human and rat ET_B-R gene, respectively. (ET_B-R mRNA abundance [expressed as percentage of the mRNA in segments perfused at 0 mm Hg] in segments exposed to 20 mm Hg for 6 hours is as follows: 446-bp PCR product, 684±76%, n=23; 304-bp PCR product, 631±56%, n=23), and it occurred independently of the presence of an intact endothelium (Figure 4c). Basal ET_B-R expression also did not differ significantly between denuded and endothelium-intact segments (122±9% of the mRNA abundance in denuded segments, n=5).

There are no suitable antibodies for rabbit ET_A-R and ET_B-R (Western blot analyses that used two different anti-peptide antibodies raised in sheep [BioTrend, Research Diagnostics Inc] and an anti-peptide antibody directed against the intracellular C-terminus raised in rabbits [courtesy of Dr C. Schröder, Institute for Physiological Chemistry and Pathobiochemistry, University of Mainz, Mainz, Germany] failed to detect a protein band of the expected size, 47 to 49 kDa, in homogenates of the rabbit jugular vein.) Because of this lack

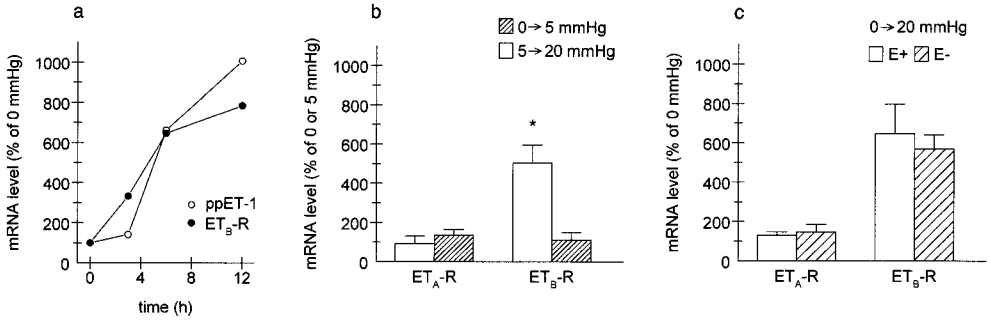

Figure 4. a, Time-dependent increase in ppET-1 and ET_B-R mRNA abundance in E+ segments perfused at 20 mm Hg. The figure shows two representative experiments with segments obtained from the same animal. b, Changes in ET_A-R and ET_B-R mRNA abundance (expressed as percentage of the mRNA level in segments perfused at 0 to 1 or 5 mm Hg) in E+ segments after raising the perfusion pressure from 0 to 1 to 5 mm Hg for 6 hours or, after a 3-hour equilibration period at 5 mm Hg, from 5 to 20 mm Hg for 6 hours (double determination, n=3). *$P<0.05$ vs 0→5 mm Hg. c, Effects of 6 hours exposure to 20 mm Hg on the level of ET_A-R and ET_B-R mRNA in E+ and E− segments (n=5).

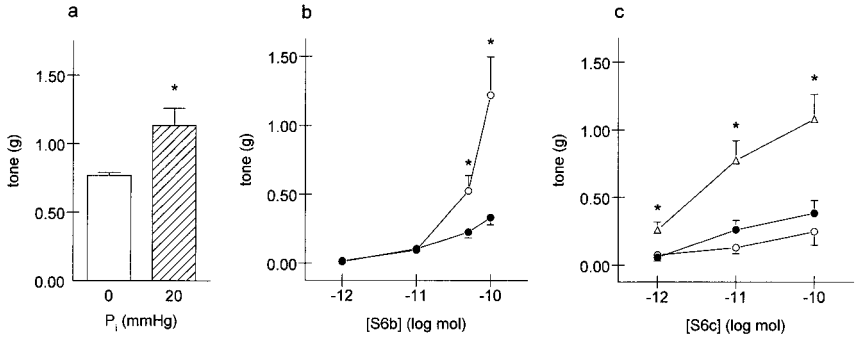

Figure 5. a, Pressure-induced (20 mm Hg, 6 hours) increase in the constrictor response to 0.1 nmol ET-1 (n=26, *P<0.05 vs 0 to 1 mm Hg). b, Pressure-induced (20 mm Hg, 6 hours, ○, n=5 to 10) increase in the constrictor response to S6b, ●, n=7 to 11 (*P<0.05 vs 0 to 1 mm Hg). c, Pressure-induced (20 mm Hg, 6 hours, ○, n=12; 20 mm Hg, 3 hours, Δ, n=8) changes in S6c-induced contractions, ●, n=22 (*P<0.05 vs 0 to 1 mm Hg). All experiments were performed with E+ segments.

of suitable antibodies for rabbit ET_A-R and ET_B-R and the rather large amounts of protein required for receptor binding assays, the superfusion bioassay method was used to confirm that in addition to the pressure-induced increase in ET_B-R mRNA, there is a corresponding increase in functional receptor protein. To this end, we determined whether the constrictor response to ET-1, the mixed receptor agonist,[14,15] sarafotoxin 6b (S6b), or the specific ET_B-R agonist,[14,15] sarafotoxin 6c (S6c), differs between segments exposed to a perfusion pressure of 0 and 20 mm Hg for 3 to 6 hours. The threshold dose for ET-1–induced constriction of these segments was 30 pmol, irrespective of the treatment. Both sarafotoxins elicited a constrictor response, the magnitude of which was comparable to that of ET-1 (Figure 5). The constrictor response to all 3 agonists up to a dose of 0.1 nmol (corresponding to a final concentration of ≈100 nmol/L in the superfusate) was almost completely abrogated (>93% inhibition, n=3) by superfusion of the segments with the ET_B-R–specific antagonist,[13,14] BQ 788 (1 μmol/L), whereas the ET_A-R–specific antagonist, BQ 123(1 μmol/L), produced only a weak inhibitory effect (<21% inhibition, n=3). Moreover, after 6 hours exposure to 20 mm Hg, the constrictor responses to ET-1 (Figure 5a) and especially to S6b (Figure 5b) were significantly enhanced. Pressurizing of the segments for 3 hours also markedly augmented the constrictor response to S6c, whereas there was no such difference after 6 hours exposure to 20 mm Hg (Figure 5c). This increased efficacy of S6c after 3 hours exposure to 20 mm Hg did not differ between endothelium-intact and -denuded segments (not shown).

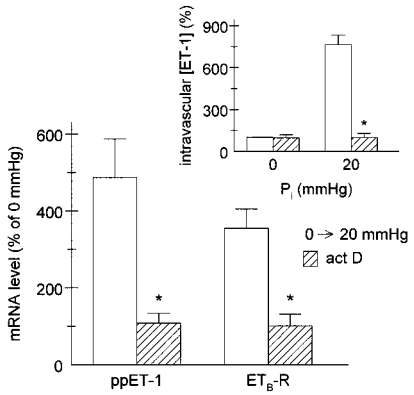

Figure 6. Effects of actinomycin D (act D, 1 μmol/L) on the pressure-induced (20 mm Hg, 6 hours) upregulation of ppET-1 and ET_B-R mRNA (calculated as percentage of the mRNA abundance in the corresponding control segments perfused at 0 to 1 mm Hg) in E+ segments (n=5 to 7). *P<0.05 vs control. The insert depicts the effects of act D on the intravascular concentration of ET-1 in segments exposed for 6 hours to a perfusion pressure of 0 to 1 or 20 mm Hg, respectively (n=3 or 4). *P<0.05 vs control.

Regulation of Pressure-Induced Gene Expression at the Transcriptional Level

Blockade of RNA synthesis with actinomycin D (1 μmol/L) completely abrogated the pressure-induced increase in prepro-ET-1 and ET_B-R mRNA (Figure 6) as well as the pressure-induced increase in intravascular ET-1 (Figure 6 insert). Basal prepro-ET-1 (77±20% of control, n=5) and ET_B-R mRNA levels (86±25%, n=5), on the other hand, were not affected by treatment with actinomycin D. The protein synthesis inhibitor cycloheximide (1 μmol/L) had no significant effect either on basal (not shown) or pressure-induced expression of these gene products after 6 hours (for prepro-ET-1, 471±101% without cycloheximide versus 312±71% with cycloheximide; for ET_B-R, 257±85% without cycloheximide versus 330±77% with cycloheximide; n=3).

Role of Protein Kinases

Pretreatment of the segments with the protein kinase C (PKC) inhibitor[15] Ro 31–8220 (0.1 μmol/L) for 1 hour had no significant effect on ECE-1 or ET_A-R mRNA levels under basal conditions and in the presence of an elevated perfusion pressure (not shown). In contrast, basal (20±9% and 6±2% of control, respectively; n=4, P<0.05) and pressure-induced (Figure 7a) prepro-ET-1 and ET_B-R mRNA levels were markedly reduced after exposure to Ro 31–8220. The putatively c-Src family–specific tyrosine kinase inhibitor, herbimycin A (0.1 μmol/L),[16,17] on the other hand, had no significant effect on prepro-ET-1 and ET_B-R abundance under basal conditions (81±11% and 58±19% of control,

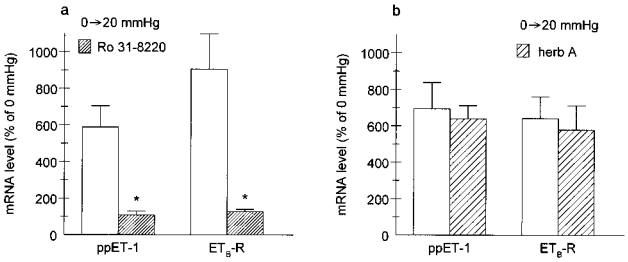

Figure 7. Effects of Ro 31–8220 (0.1 μmol/L, n=4) (a) and herbimycin A (herb A, 0.1 μmol/L, n=3) (b) on ppET-1 and ET_B-R mRNA abundance (expressed as percentage of the mRNA level in the corresponding control segments perfused at 0 to 1 mm Hg) in E+ segments exposed to a perfusion pressure of 20 mm Hg for 6 hours. *P<0.05 vs control.

respectively; n=3) and after exposure to 20 mm Hg for 6 hours (Figure 7b).

Discussion

Although the pulsatility (±1 mm Hg) and rate of perfusate flow (1 mL/min) through the isolated segments do not precisely match the conditions in the rabbit jugular vein in vivo, the newly developed model allowed us to study the effects of an enhanced perfusion pressure on gene expression in the vessel wall in situ. With the aid of this model, we were able to substantiate earlier findings in cultured endothelial cells of a stretch- and/or deformation-induced increase in prepro-ET-1 mRNA abundance[9] and ET-1 release.[7,8] The pressure-induced upregulation of prepro-ET-1 expression in the endothelium and that of the ET_B-R in the smooth muscle did not follow the pattern of an all-or-nothing response but were clearly dependent on the duration and intensity of the increase in perfusion pressure. Most important, however, these changes in gene expression did not occur at physiological pressure levels that, depending on the animal's posture and heart cycle, can be estimated for the external rabbit jugular vein at 0 to 5 mm Hg above atmospheric.[18–20]

In perfused blood vessels extended to in situ length, as was the case in our experiments, circumferential strain (ϵ) is expressed as $\epsilon=(d-d_0)/d_0$, where d is diameter, d_0 is diameter at 0 mm Hg (ie, atmospheric pressure), and $d-d_0$ is the pressure-induced change in diameter.[20] A large vein, such as the rabbit jugular vein, is maximally distensible at an intraluminal pressure of 4 to 5 mm Hg but becomes rather stiff at pressures >15 mm Hg, when collagen takes over from elastin in the vessel wall to balance the distending pressure.[18–20] Such a stiffening and collagen alignment is also observed in the human saphenous vein at arterial levels of blood pressure.[21] Monitoring by video microscopy of the outer diameter of the perfused veins confirmed that there was indeed a much greater distension between 0 and 5 mm Hg compared with 5 and 20 mm Hg. Moreover, significant changes in de novo ET-1 synthesis and ET_B-R expression were detectable only between 5 and 20 mm Hg, suggesting that the threshold for stretch-induced gene expression in venous endothelial cells and SMCs is either rather high or that these cells are deformed further by being pushed into the stiffening collagen. Irrespective of the biomechanical force ultimately being responsible for the pressure-induced increase in gene expression, the present findings clearly demonstrate that such an effect occurs only when the distending intraluminal pressure (ie, blood pressure) is clearly elevated beyond the mean circulatory filling pressure in veins (4 to 6 mm Hg).[18–20] In contrast to the situation in the rabbit and human jugular veins, the distal portions of the saphenous vein routinely used for aortocoronary bypass grafting experience much greater changes in blood pressure between 5 and 10 mm Hg in the recumbent position and between 75 and 80 mm Hg on quiet standing (ie, without activation of the muscle pump and at ambient temperature).[22] However, this hemodynamic pressure load (averaging 40 to 50 mm Hg over 24 hours)[23] is by no means as constant as after grafting of the saphenous vein into the coronary circulation, where, in addition to the mean arterial blood pressure of 90 to 100 mm Hg, the anastomosed segments are additionally subjected to a significant outflow resistance, owing to the fact that their lumen is usually much wider than that of the bypassed coronary arteries. Thus, the range of intraluminal pressures to which the isolated perfused rabbit jugular veins were subjected in the present study can be regarded as principally similar to the situation in aortocoronary venous bypass grafts.

Because the pressure-induced increase in endothelial prepro-ET-1 mRNA and in ET-1 peptide synthesis was sensitive to actinomycin D (ie, blockade of mRNA synthesis), it would appear that the elevated perfusion pressure affects prepro-ET-1 mRNA synthesis rather than stability. Despite the lack of effect on ECE-1 mRNA abundance, we cannot completely discount the possibility of an additional pressure-induced increase in ECE-1 expression or activity. Three different splice variants of ECE-1 have been described,[24] the expression of which may be differentially regulated. Our present RT-PCR protocol cannot differentiate between these splice variants, so that a note of caution may be appropriate regarding the pressure insensitivity of ECE-1 expression in rabbit blood vessels. On the other hand, the pressure-induced rise of prepro-ET-1 mRNA was accompanied by an even higher increase in the intravascular concentration of ET-1, suggesting that ECE-1 activity or expression may not be a rate-limiting factor in ET-1 synthesis in response to a pressure-dependent deformation of the endothelial cells.

In addition to the increase in prepro-ET-1 expression in the endothelium, increasing the perfusion pressure beyond 5 mm Hg also significantly upregulated ET_B-R but not ET_A-R expression in the isolated perfused rabbit jugular vein independently of the presence of an intact endothelium. This pressure-induced ET_B-R expression was confirmed at the mRNA level with two completely different PCR products obtained with primer pairs designed from the sequences of the human and rat ET_B-R gene, respectively. Moreover, it appeared to be controlled at the level of transcription, in view of the fact that it was abolished by actinomycin D.

To demonstrate that the observed pressure-induced increase in ET_B-R mRNA also translated into an increase in ET_B-R protein, the superfusion bioassay technique that enabled us to measure specific alterations in endothelin receptor-mediated responses was used. We could confirm previous findings of the rabbit jugular vein behaving as a pure ET_B-R preparation,[13,14] even in the presence of S6b, which may be explained by the known lack of selectivity of this receptor for ET-1, ET-2, ET-3, and the sarafotoxins. However, there was a difference between the two sarafotoxins that might be related to a higher efficacy of S6b compared with S6c in the presence of endogenously synthesized ET-1. Thus, the pressure-induced potentiation of ET_B-R–mediated vasoconstriction (indicative of an increase in ET_B-R protein abundance) after 6 hours exposure to 20 mm Hg was observed with S6b but not S6c. However, when the segments were pressurized for 3 hours (ie, when the pressure-induced increase in prepro-ET-1 mRNA abundance was still rather small; see Figure 4a), the potentiation of the constrictor response to S6c was comparable to that of S6b after 6 hours exposure to 20 mm Hg. Therefore, it may be that the ET_B-R is rapidly desensitized by endogenous ET-1[25] and that this desensitization (or shift in affinity) can be overcome by S6b but not S6c. Notwithstanding these considerations, the superfusion bioassay data demonstrate that the pressure-induced increase in ET_B-R mRNA abundance in the isolated perfused

rabbit jugular vein is accompanied by an increase in functional ET_B-R protein.

Circumferential strain has been linked to gene expression in vascular cells by two major signaling pathways that may join at the level of Raf kinase, ie, the phospholipase C–PKC and the c-Src–Ras pathway.[26] In a first attempt to elucidate the signal transduction pathway involved in pressure-induced gene expression, Ro 31–8220, a highly selective inhibitor of PKCα, PKCβ_I, PKCβ_{II}, PKCγ, and PKCϵ with IC_{50} values ranging from 5 to 27 nmol/L,[15] was used. Ro 31–8220 completely abrogated the pressure-induced increase in prepro-ET-1 mRNA abundance, supporting previous results of a PKC-mediated release of ET-1 from cultured endothelial cells in response to cyclic strain.[9] Moreover, PKC blockade also abolished the pressure-induced rise in ET_B-R mRNA. In contrast, exposure of the segments to herbimycin A had no such effect. At the concentration used (100 nmol/L), herbimycin A appears to be highly specific for the c-Src family of tyrosine kinases and does not affect the activity of other tyrosine kinases.[16,17] It would appear, therefore, that in endothelial cells and in SMCs of the rabbit jugular vein, activation of one or several isoforms of PKC is crucial for the pressure-induced increase in gene expression.

In addition to providing a basis for a more detailed investigation of the signaling mechanisms involved in pressure-induced gene expression in the vessel wall in situ, the aforementioned findings raise the question as to what the functional consequences of the upregulation of ET-1 synthesis and ET_B-R expression in response to a maintained suprapysiological pressure level are. One distinct possibility is that this may lead to excessive vasoconstriction and hence to the vasospasm that is frequently observed in acute graft failure, especially because ET_B-R–mediated vasoconstriction is usually observed in veins only.[5] Moreover, venous but not arterial SMCs in culture have been reported to proliferate in response to cyclic strain.[27,28] This effect could also be triggered by ET-1 via the ET_B-R[29] and thus contribute to the intimal hyperplasia of venous bypass grafts, which in up to 35% of patients with coronary heart disease causes their failure 1 year after the surgical intervention.

However, there are other reports of an increased ET_A-R expression in porcine saphenous vein–carotid artery interposition grafts 1 month after surgery,[30] a downregulation of ET_B-R in rabbit saphenous vein-carotid artery interposition grafts 1 month after surgery,[31] and no apparent change in ET_A-R and ET_B-R distribution but an enhanced ET_A-R–mediated sensitivity to ET-1 in human aortocoronary saphenous vein grafts several years after surgery.[32] In a related setting of pressure-induced gene expression (ie, angioplasty), on the other hand, evidence has been provided that points to an important role for the ET_B-R or a non–ET_A-R/non–ET_B-R in the rabbit[33] and rat carotid artery[34–36] in restenosis. Therefore, further studies in an appropriate animal model (ie, jugular vein–carotid artery interposition graft) are required to substantiate the hypothesis of an ET-1–induced ET_B-R–mediated proliferative response in aortocoronary venous bypass grafts. The timing of these experiments may turn out to be crucial because the pressure-induced increase in smooth muscle ET_B-R expression in the rabbit jugular vein appeared to be a transient phenomenon.

In summary, the aforementioned findings reinforce the notion of a pressure-induced rise in endothelial ET-1 synthesis along with an increased expression of the ET_B-R in the smooth muscle of the rabbit jugular vein. Provided that such blood pressure–induced changes in gene expression also occur in the human saphenous vein in vivo, they may well contribute to the acute or intermediate failure of aortocoronary venous bypass grafts. Therefore, it may be advantageous to reevaluate the therapeutic benefit of a selective ET_B-R or a mixed ET_A-R/ET_B-R antagonist in these conditions.

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